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The role of propionate in cellular metabolism.

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THE ROLL PROPIONATE IN CELLULAR METABOLISM

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THE ROLE OF PROPIONATE
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CELLULAR METABOLISM

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Gordon C. Bell

Letter on cover:

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THE ROLE OF PROPIONATE IN CELLULAR METABOLISM

by

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Submitted in partial fulfillment
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ACKNOWLEDGEMENT.

This report is a representative survey of the literature on the subject to the present date, and is by no means a complete reference; over 200 original references were read and abstracted.

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PREFACE

Microorganisms offer enormous advantages over almost any other type of biological material for the study of comparative biochemical problems. The reason can be found in the fact that, in many cases, their metabolic processes are so much more clearcut than those in the higher plants and animals, as well as in the possibility of influencing these reactions by very simple procedures in a readily interpretable manner. It is indeed fortunate for the purpose of comparing certain metabolic processes that we find peculiarly exaggerated types of metabolism among microorganisms. [20]

When a bacterium is inoculated into a nutrient medium, it first begins to increase in size, and this increase in cell material is eventually followed by binary fission. The speed at which this process takes place depends on the particular organism concerned and on the physico-chemical constituents of the environment. Escherichia coli, a common organism of study, will divide once every twenty minutes when inoculated into a nutrient broth at pH 7 and 37°C. This means that each cell synthesizes its own weight of protoplasm, including proteins, enzymes, prosthetic groups, essential metabolites, etc., in twenty minutes.

For synthesis to occur an organism requires (1) inorganic salts, (2) a source of carbon, (3) a source of nitrogen, and (4) a source of energy. Some organisms can synthesize all their

protoplasm from simple sources such as carbon dioxide and ammonia, plus a source of energy, and must therefore be equipped with all the enzymes necessary for the formation of the essentials of their existence from these simple sources. This is not the case with all bacteria, as many organisms are lacking in enzymes necessary for certain synthetic processes. When this occurs, the organism in question is unable to synthesize some essential constituent and is consequently unable to grow unless and until that particular constituent is supplied ready made in the environment. When an organism has such a synthetic disability it is said to be nutritionally "exacting" towards the substance which it is unable to synthesize.

The least exacting group of organisms is the autotrophic group, members of which are able to multiply in a purely inorganic environment, synthesizing their carbon substances from CO_2 or HCO_3, their nitrogenous material from ammonia or nitrate, and obtaining their energy in one of two ways: /9/

CHEMOSYNTHETIC AUTOTROPHS: Certain organisms synthesize all their protoplasmic materials from CO_2 or HCO_3 and NH_3 or NO_3, and obtain the energy for the synthesis by oxidation of an inorganic substrate which is specific for the particular organism, and by means of which the organism can be identified.

PHOTOSYNTHETIC AUTOTROPHS: The photosynthetic autotrophs are anaerobes and obtain the energy for their synthetic activities by photochemical utilization of light energy. They obtain

their nitrogen from ammonia or nitrate, their carbon from bicarbonate, and reduce the bicarbonate to organic carbon by a linked oxidation of an inorganic substrate. The organic carbon so produced may be of carbohydrate nature in the first instance, and can be conveniently represented by (H-C-OH). The Athiorhodaceae [14] use organic acids as hydrogen-donators whereby to reduce the CO_2. CO_2 is essential to the growth and the organism appears to obtain most of its carbon material from the assimilation of CO_2:

In contrast to the autotrophic bacteria are the HETEROTROPHIC organisms which require a source of carbon more complex than carbon dioxide, i.e., they are unable to utilize carbon dioxide as a sole source of carbon. However, heterotrophic organisms actually require trace amounts of CO_2, only as a source for certain essential carbon compounds. In the fermentation of many organisms, such as E. coli or the Propionibacteria, carbon dioxide assimilation is involved in the formation of succinic and other 4-carbon dicarboxylic acids.

Studying the production of propionic acid from sugars is equivalent to studying its conversion to sugars, since one process is probably the exact reverse of the other, involving the same intermediate compounds and enzymes.

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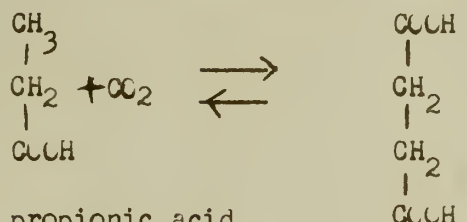
CHAPTER I

INTRODUCTION

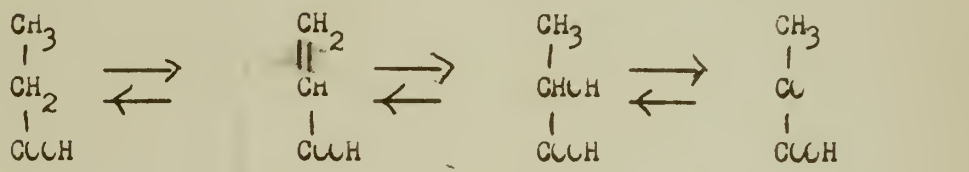
In contrast to other simple compounds, the metabolism of propionic acid is poorly understood. Some probable pathways are:

(I)



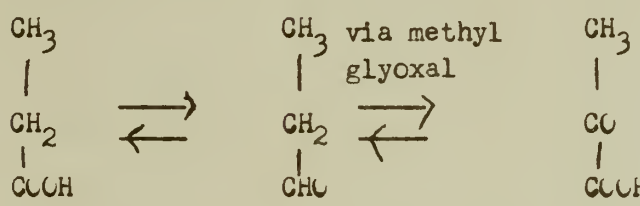
propionic acid — succinic acid

(II)



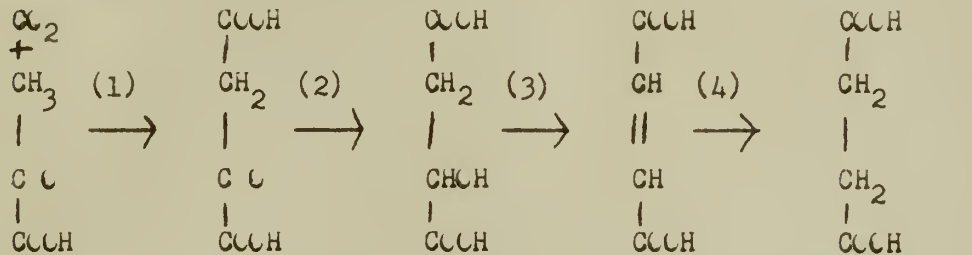
propionic acid — acrylic acid — lactic acid — pyruvic acid

(III)



propionic acid — propionaldehyde — pyruvic acid

Experimental support of one or another of these schemes may be uncertain because of possible interconversion, demonstrated in most organisms, of succinic and pyruvic acids:



pyruvic acid	oxaloacetic acid	malic acid	fumaric acid	succinic acid

(1) Oxalacetic decarboxylase

(2) Malic dehydrogenase

(3) Fumarase

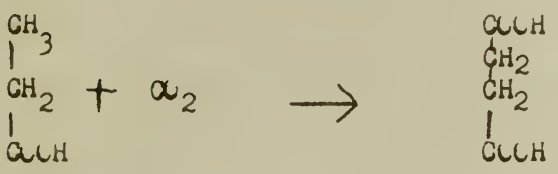
(4) Succinic dehydrogenase

We shall not concern ourselves with the subsequent fate of pyruvic and succinic acids. The pathways linking these compounds to carbohydrates are well established; in any case, such questions are beyond the scope of the present study. Thus, for example, if an organism produces propionic acid from glucose it is assumed that pyruvic acid is an intermediate, formed through the conventional glycolysis cycle.

The formulation of a rigid pathway for the production of the end products is highly improbable, because the biological breakdown of sugars and related substances by bacteria probably takes place by more than one mechanism. [26]

CHAPTER II

INVESTIGATIONS FAVORING THE PATHWAY



propionic acid succinic acid

H. Larsen [14] found the photosynthetic purple bacteria are characterized by their ability to utilize simple organic compounds, particularly the lower fatty acids, as carbon sources in photosynthesis. They grow profusely under anaerobic conditions in the light in a mineral medium containing propionate, and the amount of cell material synthesized is determined by the amount of propionate available. It has been shown that the conversions taking place can be approximately expressed by the following simple equation:

$$CO_2 + 2\ CH_3\ CH_2\ COO\ Na + 3H_2O \xrightarrow{\text{light}} 7\ (CH_2O) + 2\ Na\ OH$$

In the absence of light no cell synthesis takes place under anaerobic conditions.

Resting cells of a recently discovered photosynthetic green sulfur bacterial species, Chlorobium thiosulfatophilum, were found to bring about a light dependent conversion of propionic acid and CO_2 to succinic acid.

Larsen observed that C. thiosulfatophilum does not give the reaction shown above, for the following reasons: (a) C. thiosulfato-

philum does not grow at the expense of propionate; $Na_2S_2O_3$ was required; (b) there was a decrease in bicarbonate of suspensions illuminated in the presence of propionate and CO_2, demonstrating the formation of acidic products; (c) there was a 1:1 relationship between the number of moles of CO_2 assimilated and of propionate added.

Two different reaction paths should be considered. One involves a dehydrogenation of propionic to pyruvic acid, and subsequent reductive carboxylation to malic acid, followed by a further reduction to succinic acid. An alternative reaction path involves a carboxylation of propionic acid itself, or some biologically activated form of propionic acid, to give succinic acid. This reaction has never been experimentally demonstrated in a biological system but has been suggested in a few cases. However, the reverse of this reaction, i.e., a direct decarboxylation of succinic acid to form propionic acid and CO_2 has been presented as an explanation for the mechanism of formation of propionic acid by propionic acid bacteria and certain micrococci.

It is possible that the mechanism by which CO_2 is fixed into succinic acid is different from the one by which CO_2 is fixed into cell material in normal photosynthesis, or that it represents but one of several CO_2 fixing mechanisms. Larsen ran an experiment in which C. thiosulphatophilum was allowed to photosynthesize under light-saturating conditions in the presence of both thiosulfate

and propionate. In this case the rate of CO_2 fixation was equal to the sum of the rates observed with each of the two hydrogen donors separately. This suggests that the assimilation mechanisms do not proceed by way of a common assimilation product.

The light-dependent formation of succinic acid from propionic acid and CO_2 represents from a biochemical point of view, a much simpler type of photo-synthesis than the usual light-dependent conversion of CO_2 into the poorly defined "cell material." The new system might therefore be of value in exploring biochemical mechanisms by which CO_2 can be built into organic compounds by means of light energy.

Delwiche grew cultures of *R. pentosaceum* for 36 hours at 30°C after a 5 per cent inoculation into a 4-liter Erlenmeyer flask containing 2 liters of a medium composed of 0.5 per cent each of glucose, peptone, and Difco yeast extract. At the end of the incubation period the cells were removed by centrifugation, washed twice in a volume of distilled water equal to the original volume of the fermentation broth, and resuspended in phosphate buffer of pH 5.2 or in distilled water. 76/

All the experimental work concerning rates of fermentation of pyruvate, succinate and other substrates was conducted by means of the usual Warburg manometric techniques. A total of 3.0 ml. was suspended in each Warburg vessel and was composed of 1.0 ml. of a suspension of cells in m/100 phosphate buffer of pH 5.2, 0.2 ml. of m/5 substrate, and, if succinate was the substrate, 0.3 ml. of m/10

semicarbazide sulfate. The total volume in all cases was made up to 3.0 ml. by the addition of m/100 phosphate buffer of pH 5.2. Incubation was at 30° C. for 120 minutes under an atmosphere of nitrogen. Activity was expressed as uL of CO_2 per mg. per hour, hereafter designated as Qco_2.

Both pyruvate and succinate were found to be decomposed at significant rates (Qco_2 of 11.1 and 4.4, respectively, for the first 60 minutes when 35 mg. of cells were present). The semi-carbazide was included as a ketone trapping agent to eliminate the possibility of reversion from succinate to pyruvate.

A similar experiment was conducted in order to determine the amounts and proportions of volatile acids produced and the carbon dioxide evolved from pyruvate as compared to succinate. The experiment was identical to the one concerning rates with the exception that the incubation period was increased to 240 minutes and the vessels utilized were six with pyruvate as a substrate and six with succinate as a substrate, plus six control cups, for the purpose of making endogenous corrections of the analysis data. At the end of the incubation period 0.5 ml. of 5 N H_2SO_4 were added to each cup to stop the fermentation reactions, and the six cups were pooled for analysis. The pool was distilled and analyzed for volatile acids, the results of which are shown below.

TABLE I.

Volatile acids and carbon dioxide, produced from pyruvate and succinate under idential conditions.

	FROM PYRUVATE*	FROM SUCCINATE*
Acetic acid	114μ moles	6μ moles
Propionic acid	46μ moles	96μ moles
Carbon dioxide	129μ moles	108μ moles

*Values have been corrected for the following endogeneous production: 73μ M acetic acid, 38μ M propionic acid, and 34μ M CO_2.

We may note that in the decomposition of succinate, propionate, appearing quite logically in approximately equimolar amounts with carbon dioxide, is produced in significantly greater amounts than in the case of pyruvate decomposition.

Decarboxylation of succinic acid as a mode of formation of propionic acid in the genus Propionibacterium has been suggested, [25] and evidence has been presented that supports this contention. Numerous observations concerning the ability of the genus to ferment succinic acid with the formation of propionic acid have been made. [11] [7] Succinic acid substrate, fermented anaerobically, produced propionic acid and carbon dioxide in approximately equimolar amounts along with small amounts of acetic acid. The data obtained with isotopic carbon [4], [27], [28], constitute evidence that propionic acid results from the decarboxylation of a symmetrical dicarboxylic acid.

Bacterial decarboxylation of succinate has been observed [10], [11], with an anaerobic micrococcus isolated from the rumen of sheep. This organism, Micrococcus lactilyticus or Veillonella gazogenes, was strictly anaerobic and differed from the propionic acid bacteria in that it did not ferment sugars. Johns reported an optimum pH for the decarboxylation as 5.9-60, and stated that isotopic carbon from labeled carbon dioxide was fixed only in the carboxyl group of the propionic acid.

The mechanism of the conversion of propionate to carbohydrate in the animal body is unknown. This source of carbohydrate is of considerable importance to ruminants, which produce propionate by bacterial action in the rumen and absorb it from that organ. It is possible that a reversal of the steps from pyruvate to propionate could occur, involving fixation of carbon dioxide in the methyl group of propionic acid to produce succinic acid.

Barban and Ajl, using the techniques employed by Delwiche [6], and Carson et al [4], incubated $C^{14}O_2$ in the presence of inactive succinate, fumarate and malate at pH 5.8, and found there was incorporation of radioactivity into the carboxyl groups of succinate; active propionate was the major volatile end-product of the reaction. [1]

For the respiration studies conducted by Barban and Ajl, conventional Warburg methods were used. The exchange reactions were conducted in 250 ml. flasks. Separatory funnels were fitted into these vessels to permit the addition of reagents during the course of the experiments. Because the reactions were to be carried out at acid pH,

and since the solubility of CO_2 in acid solutions is small, the following procedure was adopted in performing the experiment. After mixing the cell suspension with the appropriate substrate, the reaction vessels were flushed with a mixture of 95 per cent nitrogen and 5 per cent CO_2 for 10 to 15 minutes. At this point $NaHC^{14}O_3$ solution contained in the separatory funnel was added by opening the stopcock and forcefully blowing into the funnel. This procedure assured a saturated $C^{14}O_2$ atmosphere in the reaction vessel.

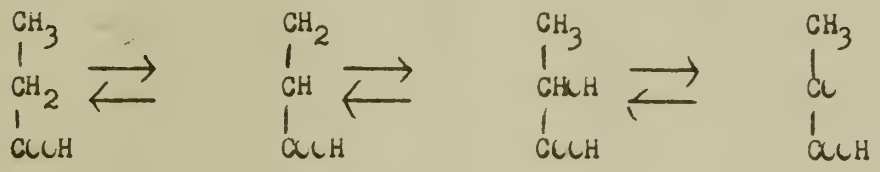
At the end of the incubation period, the reaction mixture was acidified and the proteinaceous material removed by centrifugation. The supernatant solution containing the water-soluble components of the reaction was steam distilled to remove volatile acids, while the residue was ether-extracted for 24 to 48 hours to remove the dicarboxylic acids.

The steam distillate was neutralized and boiled down to a small volume. A portion was then put on a sheet of filter paper and chromatographed, with a mixture of propanol, ammonium hydroxide and water in 6:3:1 proportions, respectively. After spraying the sheets with bromcresol purple, the bands corresponding to acetate and propionate were eluted and quantitatively determined by titration with 0.08n NaOH. The identity and concentration of these acids were also determined by the partition method [21].

The results of several experiments strongly suggest that the $NaHC^{14}O_3$ carbon in the succinate entered via the succinic carboxylase enzyme system described by Delwiche.

CHAPTER III

INVESTIGATIONS FAVORING THE PATHWAY



propionic acid — acrylic acid — lactic acid — pyruvic acid

Wood and Leaver [16] have found that if a cleavage of the 4- or 5-carbon substrates to C_1 or C_2 compounds occurs, these cleavage products are largely converted to succinate and propionate by secondary reactions. Under certain conditions, it was found that the CO_2 turnover was much lower than that required by a mechanism in which succinate is decarboxylated to propionate and CO_2. Leaver and Wood, [26], suggest that the formation of propionate from a C_4 dicarboxylic acid may involve a "C_1" which is not CO_2, but may be converted to CO_2. There is evidence that propionate can be formed by certain organisms without the occurrence of a C_4 dicarboxylic acid. Cardon and Barker [3] and Johns [10] have presented results with Clostridium propionicum that indicate there is a direct reduction of lactate to propionate, possibly via acrylate. Leaver [15] has provided additional evidence supporting this possibility in that the fermentation of lactate -3-C^{14} by C. propionicum led to almost quantitative formation of propionate-3-C^{14}.

Wood and Leaver [16] state that their observations indicate that:

(1) there is a biolcgical mechanism for formation of propionate by direct reduction; (2) in the propionic acid fermentation, the C_2 turnover is too low to indicate that all the propionate is formed by decarboxylation of succinate to propionate and C_2; (3) there is a mechanism for randomization of the 2-and 3- positions of propionate, and (4) there is evidence that a C_1 other than C_2 may be formed in the conversion of succinate to propionate.

Leviton and Hargrove [18] investigated the possibilities of commercial fermentation of lactic acid to propionic acid, as suggested by profitable amounts of vitamin B_{12} as a by-product.

Wood et al [17] state that glucose fermentations by Propionibacterium arabinosum and P. shermanii demonstrate that the formation of propionate solely from succinate is unlikely, and that the two species may actually have different mechanisms of glucose metabolism. Leaver et al [20] used Clostridium propionicum and found that lactate-3-C^{14} was reduced directly to propionate-3-C^{14}; when lactate-3-C^{14} is fermented by P. arabinosum, the distribution of tracer C^{14} in the propionate suggests that succinate can be the precursor. It is suggested that succinate and propionate can be formed in part by a condensation reaction, perhaps via citrate.

CHAPTER IV

INVESTIGATIONS FAVORING OTHER SCHEMES.

Before about 1935 it was believed that the succinic acid formed by the propionic acid bacteria arose either from the hexose molecule being split into C_4 and C_2 compounds, from acetic acid, or from the nitrogenous constituents of the medium. In that year Wood and Werkman [25] began their studies on the source of succinic acid, which is formed from glycerol by the propionic acid bacteria. Later studies led them to assume that the succinic acid is actually synthesized from pyruvic acid and C_2. This assumption has since been shown to be correct as the result of studies using isotopic carbon in C_2 and demonstrating its presence in succinic acid [26], [27].

Wood and Werkman postulated the following reaction, using P. arabinosum and glycerol as a substrate.

$C_6H_{12}O_6 \longrightarrow 2\ CH_3COCH(OH)_2 \longrightarrow$

glucose — methyl glyoxal hydrate

methyl glyoxal — propionaldehyde

$$\begin{array}{ccc} CH_3CHOHCHO & \longrightarrow & CH_3CH_2CHO \\ +H_2O \downarrow -2H^+ & & +H_2O \downarrow -2H^+ \\ CH_3CHOHCOOH & & CH_3CH_2COOH \end{array}$$

Propionaldehyde was isolated as an intermediate; several other

investigators [28], [16], have tried to obtain similar results, without success.

Other investigators [13] have postulated that the formation of propionate from pyruvate has the following reaction:

$$3\ CH_3\ CO\ COOH + H_2O \xrightarrow[\text{cycle}]{\text{Krebs}} 2\ CH_3\ COOH + CH_3\ CH_2\ COOH + 2\ CO_2$$

From the data presented, it appears that the succinic decarboxylase system is sufficiently active to produce propionic acid from succinate at a rate comparable to the rate of production of propionic acid from pyruvate.

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